Screening and Identification of Ovulation-Inducing Regulatory Factors in Bactrian Camel

Qi Wang (✉ forever45214125@126.com)

Gansu Agricultural University

Quan wei Zhang

Gansu Agricultural University

Ze Gan

Gansu Agricultural University

Hai jiang Li

Gansu Agricultural University

Yi na Li

Gansu Agricultural University

Xing xu Zhao

Gansu Agricultural University

Yong Zhang

Gansu Agricultural University

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Abstract

Background: Camelidae are inducing ovulators, ovulation is tightly regulated by multiple factors, and understanding the biological mechanisms underlying follicular development, hormone secretion and ovulation requires investigation of the potential molecular pathways. However, little is known about the pathways of these factors in the camel. To screening and identification candidate biomarkers after inducing ovulators in ovary.

Methods: In this paper, the quantitative proteomics method iTRAQ analysis technology was used to analyze the proteomic differences of ovarian before and after induced ovulation in Bactrian camels. The differentially expressed protein was validated by Western blot, qRT-PCR and Immunofluorescence staining analysis.

Results: A total of 5,075 ovarian expressed proteins were detected, and 404 proteins were differentially expressed (264 up-regulated, 140 down-regulated) in samples from treated versus control camels. Gene ontology annotation identified potential functions of the differentially expressed proteins (DEPs). We validated the differential expression for a subset of these proteins using western blotting and immunofluorescence staining. Three DEPs (FST, NR5A1 and PRL) were involved in neurochemical signal transduction, endocrine and reproductive hormones regulatory processes. KEGG analysis indicated the involvement of several pathways, such as calcium, cAMP, gonadotropin-releasing hormone, MAPK, and neuroactive ligand-receptor signaling pathways, further suggesting that the induced ovulation process depends on the hypothalamic-pituitary-ovarian axis.

Conclusions: This study is the first to perform quantitative proteomic investigation by iTRAQ labeling and LC-MS/MS to identify differentially expressed proteins induced ovulation in Bactrian camels. Our study has revealed distinct molecular functions and metabolic pathways that are active during reproduction in Bactrian camels. These results have demonstrated the pivotal role played by 3 DEPs in the modulation and activation of the reproductive axis during induced ovulation in the Bactrian camel, followed by the measurement of selected proteins using more targeted methods, offers a promising approach for studying potential mechanism of ovary development and ovulation.

Background

The Bactrian and dromedary camel, alpaca, llama, guanaco and vicuna, belong to the family Camelidae [1]. Camels live in extreme desert environments in Africa and Asia, and their adaptations to arid conditions include hydropenia and a tolerance of temperatures exceeding 40°C [2, 3]. Gonadotropin releasing hormone (GnRH) and luteinizing hormone (LH) are critical regulatory factors of the neuro-endocrine system controlling ovulation [4]. Two categories of ovulation, spontaneous and induced, have been classified in species based upon the release of GnRH/LH. In spontaneous ovulators, such as cattle, goats, horses, monkeys, pigs, sheep, and most rodents, perennial or seasonal estrus leads to regular ovulation. In induced ovulates, such as alpaca, Bactrian camels and dromedary camels, cats, ferrets,

llama and rabbits, neural signals elicited by the act of copulation trigger hypothalamic GnRH secretion, which induces preovulatory LH release from the pituitary gland [5]. Although different stimuli (olfactory, tactile, and visual) have been associated with eliciting/facilitating ovulation in induced ovulators [6, 7], the physical stimulation of penile intromission or cervical stimulation plays a pivotal role in triggering the LH surge and subsequent ovulation [8]. Studies have shown that llama seminal plasma contain an active OIF (ovulation-inducing factors), which is similar to GnRH [9]. Zhao et al. performed studies involving the isolation and purification of ovulation-inducing factors from semen. A single intramuscular or intravaginal administration of male SP (seminal plasma) induced an LH surge followed by ovulation [10]. Other studies compared the effects of intravaginal administration of fresh and frozen semen, SP, washed spermatozoa, and the intramuscular injection of SP, on the ovulation rate in Bactrian camels. This work showed that ovulation occurred in most females inseminated with whole semen, independently of whether the semen contained frozen spermatozoa or not [11].

Studies have isolated and purified an OIF from other camelids (alpacas and llamas) [12], and determined the protein structure of this OIF by X-ray crystallography [13]. Other research found that camel OIF directly or indirectly regulated ovulation through the hypothalamic-pituitary-gonadal (HPG) axis [14]. However, the induction of ovulation in the Bactrian camel is extremely complicated, involving coordinated regulation by the endocrine, reproductive and nervous systems, and also by the coding and regulation of genetic information. The mechanism of induction of ovulation in Bactrian camels remains unclear. Therefore, this study used iTRAQ technology to screen ovulation-inducing proteins via bioinformatics, and then verified key target proteins by PCR, immunoblotting and immunofluorescent staining, to identify potential molecular regulatory mechanisms. This work provides the foundation for future research into the induction of ovulation, and

reproductive potentials in Bactrian camel.

Materials And Methods

Animals and handling conditions

The study was conducted at the breeding base farm in Zhangye City. Nine Bactrian camels (three males and six females) were kept in pasture and provided supplementary hay, feed pellets and Italic water.

All samples were collected in strict accordance with the ethical guidelines approved by the Animal Care Commission of the College of Veterinary Medicine, Gansu Agriculture University (with ethical approval number: GSAU-AEW-2018-0128).

Semen collection and seminal plasma preparation

Semen was collected from three mature male Bactrian camels (5 to 7 years old) two to three times a week over a period of 3 months using an artificial vagina inserted into a wooden phantom, as previously described [15]. A total of 60 ejaculates were obtained, with an average volume of 4 mL per ejaculate.

Briefly, ejaculates were centrifuged at 1500 x g for 30 min to remove spermatozoa. Sperm-free SP was pooled in 5–6 mL aliquots and stored at – 20°C. A drop of supernatant was evaluated by microscopy to confirm the absence of cells. If spermatozoa were detected, the sample was centrifuged and evaluated again until no spermatozoa were detected. Sperm-free samples were stored at – 80°C.

Seminal plasma injected, and sample collected.

The 5-7year-old females used in experiments were examined daily by manual touch and transrectal ultrasonography (TRUS) using an ultrasound scanner (MyLab 30, Esaote; Maastricht, The Netherlands). Bactrian camels with growing preovulatory size follicles (8–14 mm) were assigned to two groups; (1) intramuscular injection of 5 mL phosphate buffered saline (PBS, n = 3, control); (2) intramuscular injection of 5 ml SP (n = 3, treated). After injection, blood was collected every 4 h and follicular development was detected by TRUS. After 48 h, the camel was slaughtered, and samples were collected.

Samples preparation and collection

The samples (hypothalamus, pituitary, pineal, ovary, uterus and oviduct) were obtained immediately after slaughtering (3 treated groups and 3 control groups), partially samples fixed for histomorphological observation, other samples were stored in liquid nitrogen. Subsequently, samples were immediately stored at -80°C.

iTRAQ labeling and SCX fractionation and LC-electrospray ionization MS/MS

200 µg of peptide mixture from each ovarian sample was labeled with iTRAQ reagent, according to the manufacturer's instructions (Applied Biosystems).

iTRAQ labeled peptides were fractionated by SCX chromatography using the AKTA Purifier system (GE Healthcare). LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Scientific) coupled to an Easy nLC (Proxeon Biosystems, now Thermo Fisher Scientific), for 60/120/240 min (times determined in a pilot study). The mass spectrometer was operated in positive ion mode. MS data were acquired using a data-dependent top10 method, with the most abundant precursor ions from the survey scan (300–1800 m/z) being dynamically chosen for HCD fragmentation. The experimental process and the data analysis were carried out as previously described [16].

MS/MS spectra were searched using MASCOT engine (Matrix Science, London, UK; version 2.2) embedded into Proteome Discoverer 1.4 (Thermo Fisher Scientific Inc. San Jose, CA, USA). The following parameters were set. Brief, peptides were matched against Bactrian camel database in NCBI with peptide mass tolerance 20 ppm, MS/MS tolerance 0.1 Da, enzyme trypsin, missed cleavage 2, fixed modifications carbamidomethyl (C), iTRAQ 8-plex (K), and iTRAQ 8-plex (N-term), variable modification oxidation (M), search thorough, and false discovery rate b 0.01. Protein ratios were calculated as the median of only unique peptides of the protein quantification. Normalizes all peptide ratios by the median protein ratio, the median protein ratio should be 1 after the normalization. The ProGroup algorithm was used to

eliminate redundancy. The P value is calculated using t-test. Statistical analysis was performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA).

Hormone analysis

Peripheral blood was collected at 0, 4, 8, 12, 24, 36 and 48 h, and serum was isolated to measure hormone levels; The estradiol (E2), Follicle-stimulating hormone (FSH), Gonadotropin releasing hormone (GnRH), Luteinizing hormone (LH), progesterone (PROG) and prolactin (PRL) assayed content was measured using RIA methods, this was followed by the essentially according to Greenwood et al [17]. In short, the serum samples were further incubated at 37°C for another 1-1.5 h, add hormone radioactive marker and the incubation continued for an additional period of 1-1.5 h, and the incubation continued for an additional period of 10–12 h; at the end of this period, the precipitate was harvested and counted in a Packard Gamma Spectrometer for bound radio-activity. All samples of the various treatment groups in a particular experiment were always assayed in the same RIA in order to avoid inter-assay variations and thus enable comparisons. Each experiment in its entirety was repeated at least 3 times.

Western blot

50 µg samples were applied to a sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE, 5% + 12%) was used to confirm protein the differential expression of protein. Briefly, equal amounts ovary protein from control and treated group. Bactrian camels were resolved on SDS-PAGE gels, then transferred electrophoretically onto PVDF membranes. Membranes were then blocked for 2h at room-temperature with 5% non-fat dried milk in PBS containing 0.1% Tween 20, Next, the membranes were then incubated overnight at 4°C a with primary antibodies from mouse or rabbits (ACTB, 1:5000, Proteintech, China; CAMK2G, 1:1000, Proteintech, China; FST, 1:1000, Proteintech, China; NR5A1, 1:500, Proteintech, China; PAK1, 1:1000, Santa cruz, USA; PRL, 1:1000, Santa cruz, USA; STAG2, 1:1000, Santa cruz, USA; TGFB2, 1:500, Santa cruz, USA; Wnt2b, 1:500, Santa cruz, USA; GAPDH, 1:5000, Proteintech, China). Washed membranes were then washed three times with PBS containing 0.1% Tween 20. Washed membranes were incubated with goat anti-mouse/anti-rabbit IgG conjugated to horseradish peroxidase for 2 h at room temperature (1:5000, Bioss, Beijing, China), t and then washed three times with PBS containing 0.1% Tween 20. All immune-blot assays were performed in triplicate at minimum. Optical densities of bands were quantified, and membranes were scanned using Image-Pro plus 6.0 (Media Cybernetics Co., Rockville, USA).

qRT-PCR

Total RNA was extracted from different tissue using the TransZol Up reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

After treatment with DNase, RNA was subjected to reverse transcription using a RevertAid First-Strand cDNA Synthesis Kit (TransGen, Beijing, China), according to the supplier's instructions. The expression of the housekeeping gene GAPDH was an intra-tissue control. The primers were shown in supplementary Table S1. qPCR was carried out as reported previously [18].

Immunofluorescence staining

The staining of Hematoxylin-Eosin (HE) and Immunohistochemistry was carried out as described [19]. For immunofluorescence, after routine dewaxing and hydration, antigen retrieval in sections was conducted by microwave heating. Sections were treated with an autofluorescence quencher (Servicebio, Wuhan, China), and closed with 5% (w/v) bovine serum albumin (BSA) for 30 minutes at room temperature. Next, sections were incubated with primary antibody (1:200; Santa cruz, USA or 1:150, Proteintech, China) overnight at 4 C. After being washed with PBS, sections were incubated with FITC or ALEX conjugated goat anti-mouse IgG (1:300; Abcam, USA) at room temperature for 1-1.5 h in the dark. The nuclei were stained with 4, 6-diamidino-2-phenylindole (DAPI). Finally, the anti-fluorescence quencher was used to seal the sections. Images were observed and obtained using a fluorescence microscope (LSM800, ZEISS, Oberkochen, Germany).

Data analysis

PCR and Western blotting data were expressed as the mean $\pm$ SD using Origin 8.6 (OriginLab, CA, USA). Data were analyzed by the Student's t test (between two groups) or One-way ANOVA analysis (within multiple groups). P value less than 0.05 was statistically significant.

Results

Follicle size and serum hormone levels

Follicles of more than 10 mm diameter were collected; the average follicular diameter was 12.0 ± 1.5 mm. After 30 h, follicles in the treated group had reduced in volume, and follicle diameter decreased to approximately 7.0 ± 1.0 mm at 48 h. In the control group, follicle diameters detected by B-ultrasound were unchanged, as shown (Figure S1). Radioimmunoassay results revealed that the circulating level of LH was higher in the treatment group than in the control group, with the highest LH level at 4 h, followed by a slow decrease in the treated group compared with almost no change in the control group. The FSH level was also higher in treatment group compared with the control group and showed a slow increase with time in the treated group versus a tendency to decrease at 4 and 36 h in the control group, or a constant level at other times., Serum E2 was increased in the treated compared with control group, with the highest level at 12 h, which was significantly higher than other time points. Serum PROG was higher in the treatment group than in the control group, and the peak of PROG at 12 h was significantly higher than at other times. GnRH increased slowly from 0–24 h, with the highest concentration at 36 h. However, GnRH levels were relatively constant in the control group and did not change significantly (Fig. 1).

Protein profiling and identification of differentially expressed proteins

Ovary samples from Bactrian camels in the treated and control groups were compared using an iTRAQ experiment. we used the log2 of its fold change in expression, and the log10 of its P-value. After

processing MS/MS spectra using Mascot software, 23,786 unique peptides were mapped to 5,075 proteins. Of these, 404 DEPs were assigned to 25 different KOG categories, including “Signal transduction mechanisms” (67, 16.58%), “General function prediction only” (62, 15.35%) and “Posttranslational modification, protein turnover, chaperones” (54, 13.37%) (Fig. 2A).

Using 0.8-fold and 1.2-fold changes as the cut-off values, and $P < 0.05$, 404 proteins were found to be differentially expressed between treated and control groups; 264 were up-regulated (Table S2) and 140 were down-regulated (Table S3). GO analysis showed that certain proteins were involved in the cellular process (40.35%), metabolic process (28.96%), and biological regulation (26.98%), as well as the reproduction and reproductive process (2.23%). Proteins were also predicted to be components of cell structures (46.53 %), organelles (37.37 %), membranes (24.00 %) and synapses (1.69 %). Some were binding proteins (38.61%), while others were assigned as structural molecules (4.95%), catalytic activity (19.80%), and signal transducers (1.49%) (Fig. 2B). KEGG functional analysis indicated that the ovarian DEPs were involved in cellular processes, environmental information processing and metabolism and organismal systems, including 277 pathways. Neurological and reproductive endocrine pathways, such as the cAMP, MAPK, neuroactive ligand-receptor, phosphoinositide-3-kinase/Akt, NOD-like receptor and transforming growth factor-beta signaling pathways, were up-regulated, or downregulated to various extents (Fig. 2C). Subcellular localization analysis indicated that the DEPs are found in the cytoplasm (110, 27.23%), nuclear (106, 26.24%), extracellular (60, 14.85%), mitochondrial (56, 13.86%), plasma (40, 9.90%), cytoplasm and nuclear (14, 3.47%), cytoskeleton (8, 1.98%), endoplasmic reticulum (8, 1.98%), peroxisome (1, 0.25%), and extracellular and plasma (1, 0.25%) compartments (Fig. 2D).

Protein-protein interaction (PPI) network analysis

We grouped the 404 DEPs using fold-change of expression and then plotted their expression levels across each sample on a heat map, with red representing a significantly up-regulated protein, blue a significantly down-regulated protein, and off-white a protein with no change in expression (Fig. 3A). A total of 133 proteins were assigned to the cluster of reproduction and reproductive processes, for 5,075 identified proteins. The String online database and Cytoscape software was used to generate a PPI network of the 133 proteins associated with reproductive and reproductive processes, as demonstrated in Fig. 3B. We generated a PPI network of the 404 DEPs (264 up-regulated and 140 down-regulated in the treatment group), was developed and 100 nodes/DEPs were identified with 103 edges (Fig. 3C). Selecting 33 proteins related to the endocrine system, 19 related to reproduction and reproductive processes, and 17 proteins related to neural signal transduction, as demonstration in Fig. 3D. Further PPI analysis revealed that there were 44 nodes and 77 edges in the PPI network, which Included FST, NR5A1, PRL, CAMK2G, PAK1, PRKCD, PPP1CB, PPP1CC, STAG2 central proteins, among these, FST and NR5A1 are directly involved in steroid hormone synthesis and secretion. while PRL-NR5A1-FST mainly in PPI network; There were 4 nodes and 6 edges In PPI network, and this cluster (KRT39-KRT19-KRT17-KRT18) is associated with the estradiol receptor signaling pathway.

Expression profile verification

To verify the reliability of our iTRAQ results (Fig. 4A), we selected eight DEPs associated with reproduction, steroid hormone synthesis and secretion for western blotting analysis. Western blot results showed that GABRB2, KRT39, PAK1, PRL, STAG2 and TGF- β 2 were abundantly expressed and upregulated, and CAMK2G, CCK, FST, HSPA2, NR5A1 and Wnt2b were downregulated in ovarian tissue from the treated Bactrian camels. Western blotting data revealed that all eight DEPs showed expression patterns similar to the iTRAQ data, which demonstrating the reliability of the iTRAQ methods (Fig. 4B, C). We used multiplex immunofluorescence staining to analyze the ovarian expression pattern of eight DEPs and found that they were expressed in granulosa cells and cumulus complexes (Fig. 4E). Venn diagram constructed for the DEPs using multiple comparisons of the three groups (endocrine system, reproductive system and neural signal transduction system) (Fig. 4D). Therefore, 3 differential proteins (FST, NR5A1 and PRL) were screened, the expression patterns of 3 proteins were examined in the hypothalamic pituitary-ovarian axis and genital organs (oviduct and uterus).

Analysis of tissue expression

The expression patterns of FST, NR5A1 and PRL were analyzed via qPCR, western blotting and immunofluorescence staining. The results revealed significantly different expression levels of FST, NR5A1 and PRL mRNA in different tissues (hypothalamus, pituitary, pineal, ovary, oviduct and uterus), as well as significantly different expression levels in the same tissue from the control group compared with the treated group.

In the control group, qPCR and immunoblotting results revealed that the highest levels of FST expression were in the ovary and oviduct, with moderate expression in the pituitary and uterus, and weak expression in the hypothalamus (Fig. 5A-B). In the treated group, FST expression showed reduced expression in the pituitary relative to the control group, whereas its expression patterns in other tissues was similar to the control group (Fig. 5C-D). Immunofluorescence results suggested weak FST-immunoreactivity in of the hypothalamus, strong FST expression in the glial, pineal and pituitary cells, ovarian granulosa cells and cumulus complexes, and oviduct ciliated columnar epithelium and secretory cells, in uterine, secretory epithelial cells of endometrial gland (Fig. 6A-B).

NR5A1 mRNA expression levels were highest in the ovary, followed by oviduct and pineal expression, then weak expression in the hypothalamus, pituitary and uterus. However, western blotting results revealed strong NR5A1 expression in the ovary, oviduct and uterus, with moderate expression in the hypothalamus, pituitary and pineal cells of the control group (Fig. 5A-B). In the treated group, NR5A1 mRNA was mainly expressed in the ovary, oviduct and pineal cells, with weak expression in other tissues. However, the protein results suggested that the protein expression trend is not similar to the transcription level in hypothalamic-pituitary-gonadal axis (Fig. 5C-D). Immunofluorescence results revealed the expression levels in cell, pineal cell and glial, pineal and pituitary cells, ovarian granulosa cells and cumulus complexes, oviduct epithelial cells and endometrial cells (Fig. 6A, C).

In the control group, the highest levels of PRL mRNA and protein were observed in the pituitary. The RT-PCR results indicated weak PRL mRNA expression in the hypothalamus, ovary, oviduct, pineal gland and

uterus, however, western blotting only found strong PRL expression in the pituitary (Fig. 5A-B). In the treated group, the pituitary had the highest mRNA expression level of PRL, and the ovary and pineal had the weak expression. No protein was detected in the hypothalamus, pineal gland, oviduct and uterus (Fig. 5C-D). Immunofluorescence detected different levels of PRL expression in various tissues, the results suggest that weak PRL immunoreactivity in blood vessels, nerve cells and glial cells in the hypothalamus, weak PRL immunoreactivity in pituitary gland cells and nerve cells, strong immunoreactivity in pineal cells, variant granulosa cells, oviduct secretory epithelial cells and endometrial cells (Fig. 6A, D).

Discussion

The Bactrian camel is an induced ovulation animal, and its ovulation mechanism appears completely different from spontaneous and other induced ovulation animals. In the estrus season, the Bactrian camel follicle grows, develops and matures (diameter > 12 mm), and the female camel mates for ovulation. In llamas administered an intrauterine infusion of PBS or SP, the follicles have a diameter of 9.6 ± 0.8 mm or 8.8 ± 0.4 mm[20]. A previous study of llamas found that LH reached the highest peak 2.5 h after normal male mating, and SP reached the highest level at 4.5 h, followed by a progressive reduction [21]. In the alpaca SP, one of the most abundant proteins was identified as b-NGF by liquid chromatography mass spectrometry[22]. Many studies have shown that camel semen is rich in b-NGF, which is far more abundant in SP than found in other animals (such as bovine, horse, pig and sheep)[23]. In the present study, we using fresh SP induced ovulation in Bactrians camel, the results suggested that serum LH concentration reached the highest peak at 4 h, followed by a progressive reduction. Circulating E2 and PROG levels also increased and peaked at 12 h after mating. However, in llama females administered SP, PROG reached its highest concentration after 7 days [24]. In the current study, serum FSH and PRL levels increased significantly and were higher in the treated compared with the control group. Serum GnRH peaked at 36 h, and its level was significantly higher than the control group. Within 0–48 h, the hormone levels in the control group were almost stable. Classically, ovulation in mammals involves secretion of GnRH from the hypothalamus into the hypophyseal ovary system, followed by the release of LH from the anterior pituitary into systemic circulation [25]. the ovulatory effect of OIF in seminal plasma is mediated through a surge release of LH into blood circulation, in an study to determine the site of action of OIF, treatment of llamas with a GnRH antagonist suppressed the effects of OIF, the results suggested that a direct or indirect effect of OIF on GnRH neurons in the hypothalamus [26]. A lot of research has been done on the induction-ovulation, yet, this research still stays on the isolation and purification of OIF and hormone test. Subsequently, we were identified a total of 5,075 proteins after intramuscular injection SP, and 133 proteins were associated with reproduction and reproductive processes. The 404 DEPs (260 up-regulated, 144 down-regulated) were classified by GO analysis, and assigned into three groups (molecular functions, biological process and cellular component groups) by GO terms using multiple approaches. In addition, the up-regulated and down-regulated DEPs were further clustered based on functions and signaling pathways with significant enrichment analysis. In addition, the PPI network complex of DEPs was developed and 100 nodes/DEPs were identified with 103 edges. Finally, qPCR and Western blotting verified the expression profile of ovulation-inducing and hormone

related genes and proteins, and Immunofluorescence colocalization revealed that these DEPs were expressed in ovarian granulosa cells. Further screening of 69 DEPs related to neurochemical signal transduction, endocrine and reproductive and hormones, and mapping of the PPI network found a total of 44 nodes and 77 edges. We further screened three proteins (FST, PRL and NR5A1) using a Venn diagram. Finally, RT-PCR and western blotting verified the expression profiles of 3 proteins in HPOA. Interestingly, the most significant module was filtered from the PPI network complex, and all three proteins were located in the PPI node. The KEGG results indicated that FST, NR5A1 and PRL were involved in steroidogenic, oxytocin, follicular development and oocyte meiosis processes.

Steroid-producing factor-1 (SF-1, NR5A1) is a member of the nuclear receptor superfamily and is an orphan nuclear receptor with a genetically distinct domain different from other nuclear receptors [27]. NR5A1 is a key transcriptional regulator of genes involved in the hypothalamic–pituitary–steroidogenic axis [28]. It is expressed in tissues such as the adrenal gland, gonad, hypothalamus, pituitary, skin and spleen [29]. NR5A1 is highly expressed at sites of steroid production. This study found that NR5A1 was expressed in the fallopian tube epithelium, ovary (granulosa cells and cumulus complex), hypothalamus, pituitary, pineal gland and uterus of the Bactrian camel. KEGG analysis suggested NR5A1 involvement in the steroidogenesis process via cortisol synthesis/secretion pathways and the regulation of rate-limiting enzymes, suggesting that it may be involved in the secretion synthesis of E₂, FSH, LH, PRL and PROG. In mice, NR5A1 expression is found during the functional differentiation of steroidogenic tissues and sexual differentiation of gonads [30]. Other research found that NR5A1 was expressed in the fetal and adult adrenal cortex, as well as testicular Leydig and Sertoli cells [31]. The NR5A1 protein regulates the transcription of key genes involved in sexual development and reproduction, including STAR (steroidogenic acute regulatory protein), CYP17A1 (17- α -hydroxylase), CYP11A1 (cytochrome P-450 cholesterol side-chain cleavage) and LHB (beta subunit of LH) [32]. NR5A1 is also expressed in non-steroidogenic tissues, such as the ventromedial nucleus of the hypothalamus and in pituitary gonadotrophs [33], as well as placental venous blood vessels, sinus endothelial cells, skin and spleen [34]. In this study, camel NR5A1 was expressed at different levels in tissues of the HPG axis, as well as the oviduct and uterus, and we speculate that it may regulate the synthesis and secretion of steroid hormones.

Follistatin (FST) is a single-chain secreted glycoprotein that can bind to many members of the transforming growth factor (TGF) superfamily and inhibit their activity [35]. Earlier studies found that FST was expressed in most tissues of the body, and was highly expressed in the fetus, heart, kidney, liver, ovary and pituitary [36, 37]. Ovarian expression of FST in follicles and granulosa cells suggested that it may regulate granulosa cell differentiation through autocrine mechanisms [38]. In this study, we found that FST was highly expressed in fallopian tubes, ovary, pituitary and uterus, and weakly expressed in the hypothalamus and pineal gland of the Bactrian camel. In addition, we found differences in expression levels of FST between the treated and control groups. Other research showed that loss of FST or Wnts resulted in the loss of mouse germ cells [39]. The current work found that FST protein was mainly

distributed in glial cells of the hypothalamus and pituitary gland, the ovarian granulosa cells, and cells in uterine and fallopian tube tissue.

Prolactin (PRL) is a polypeptide hormone secreted mainly by the anterior pituitary. Carrasco et al. reported that OIF and NGF mediated ovulation and lactation in camels and cattle [40]. In this study, PRL was found in the ovary and pituitary of the treated group. In the control group, PRL was only strongly expressed in the pituitary, and was not detected in other tissues by immunoblotting. Immunofluorescence results showed a different expression level of PRL in other tissues. Previously, prolactin immunoreactivity is found within numerous hypothalamic areas in a variety of mammals and rat [41]. Placenta, Amnion, Decidua, and Uterus are also found in Rat, mouse, cow, pig, and human [42]. Some well-established stimulators, such as, ovarian steroids modulate hypothalamic E2, angiotensin II and vasoactive intestinal peptide was regulated of hypothalamic and pituitary prolactin synthesis and secretion [43, 44]. Studies have shown that PRL secretion is regulated by hypothalamic prolactin release inhibitors, prolactin releasing factors, peripheral hormones, and PRL self-feedback, to promote gonadal development and reproductive behavior [45, 46]. In addition, estrogen also promoted the synthesis, storage and release of PRL [47].

Taken together, these results have demonstrated the pivotal role played by FST, NR5A1 and PRL in the modulation and activation of the reproductive axis during induced ovulation in the Bactrian camel. However, an important future study will be to reveal the role of these DEPs in induced ovulation because Bactrians camel induced ovulation is a complicated and multifactor regulated process, of which our understanding remains incomplete. Herein, we should analyze the protein-protein interaction relationship and expound the possible roles of the critical proteins in signaling pathways enriched by DEPs. This was done to better understand the potential mechanism of ovary development and ovulation, our paper contributes to genetic breeding and improving the reproductive performance of Bactrians camel.

Conclusions

In this study, we used RIA to measure hormones and B-ultrasound to detect the development of follicles, identified differentially expressed proteins in ovary from different group using the iTRAQ labeling technique. Of the 404 proteins found to be differentially expressed. Among then, FST, NR5A1 and PRL, are known to be involved in the regulation of induced ovulation and hormone secretion. We verified the expression profiles of these three proteins by qPCR, Western blot and immunofluorescence. Our study has revealed distinct molecular functions and metabolic pathways that are active during reproduction in Bactrian camels. These results have demonstrated the pivotal role played by 3 DEPs in the modulation and activation of the reproductive axis during induced ovulation in the Bactrian camel, followed by the measurement of selected proteins using more targeted methods, offers a promising approach for studying potential mechanism of ovary development and ovulation.

Abbreviations

BSA: bovine serum albumin; DEPs: differentially expressed proteins; FSH: follicle-stimulating hormone; HE: Hematoxylin-Eosin; iTRAQ: isobaric tags for relative and absolute quantification; GnRH: Gonadotropin releasing hormone; LH: luteinizing hormone; OIF: ovulation-inducing factors; PPI: Protein-protein interaction; PRL: prolactin; PROG: progesterone; qPCR: quantitative real-time polymerase chain reaction; RIA: radioimmunoassay; SP: seminal plasma; TRUS: Transrectal ultrasonography; β -NGF: beta-nerve growth factor.

Declarations

Acknowledgements

Not applicable.

Author Contributions

Q.W., Q.Z., Z.G., H.L., Y.L., X.Z., and Y.Z. contributed to the study design, collected biological material, clinical and demographic data, and participated in writing the manuscript; Q.W. and Q.Z. conceived and designed the experiments; Q.W., Z. G., H.L., and Y.L., performed the experiments; Q.W., and Q.Z., analyzed the data; Q.W. wrote the paper; Q.Z., Y.Z., and X.Z. revised the paper. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

This study was conducted in strict accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the State Council of the People's Republic of China. The protocol was approved by the Committee on Experimental Animal Management of Gansu Agricultural University, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

¹College of Veterinary Medicine, Gansu Agriculture University, No. 1 Yingmen village, Anning District, Lanzhou, 730070 Gansu Province, China. ²College of Life Science and Technology, Gansu Agriculture University, No. 1 Yingmen village, Anning District, Lanzhou, 730070 Gansu Province, China.

Mass spectrometry proteomics data

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org>) via the iProX partner repository with the dataset identifier PXD016896 (<https://www.iprox.org/page/project.html?id=IPX0001930000>), For quantitative and analytical data, see supplementary file.

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Figures

Figure 1

Serum hormone levels (LH, FSH, estradiol (E2), follicle-stimulating hormone (FSH), gonadotropin-releasing hormone (GnRH), progesterone (PROG) and prolactin (PRL) in the treated and control groups were detected by radioimmunity assay. Data are presented as Mean $\pm$ SD (n = 3, *P < 0.05, **P < 0.01).

Figure 2

Annotation of differentially expressed proteins (DEPs). (A) Bar plot of KEGG analysis of the DEPs. (B) Gene ontology (GO) annotation and functional classification of ovarian DEPs. GO terms are given for subcellular location, biological process, cellular component and molecular function. (C) KEGG Pathway analysis for the significantly enriched ovarian DEPs. (D) Bar plot of the predicted subcellular localization of DEPs; x-axis displays subcellular structures; y-axis displays protein count.

Figure 3

Clustering and protein-protein interaction (PPI) network analysis. (A) Hierarchical clustering of differentially expressed proteins (DEPs). Clustering was based on ovarian protein expression levels in the Bactrian camel. (B) Analysis of PPI network proteins related to the reproduction and reproductive process. (C) Analysis of the PPI network for the ovarian DEPs. (D) Analysis of the PPI network for hormone and ovulation regulation related proteins in the ovarian DEPs.

Figure 4

Validation of differentially expressed proteins (DEPs). (A) Protein expression levels detected in ovary protein extracts from Bactrian camels by iTRAQ LC-MS/MS. (B) Western blot analysis of PAK1, PRL, STAG2, TGFB2, CAMK2G, FST, NR5A1 and Wnt2b in ovarian protein extracts from Bactrian camels. (C) Integrated optical density value analysis of the western blots by Image J. Data are presented as Mean $\pm$ SD; * P < 0.05, ** P < 0.01. (D) Venn diagram constructed for the DEPs using multiple comparisons of the

three groups (endocrine system, reproductive system and neural signal transduction system). (E) Immunofluorescence staining to localize the expression pattern of DEPs in ovary samples.

Figure 5

Expression and localization analysis of the differentially expressed proteins (DEPs) in the hypothalamic-pituitary-ovarian axis. (A) Expression levels of FST, NR5A1 and PRL mRNA in the control group Bactrians camel tissues, detected by RT-PCR. (B) Western blot analysis of FST, NR5A1 and PRL proteins in different tissue samples from the control group. (C) Expression levels of FST, NR5A1 and PRL mRNA in tissues from the treated group Bactrian camels, detected by RT-PCR. (D) Western blot analysis of FST, NR5A1 and PRL protein in different tissue samples from the treated group.

Figure 6

(A) H&E staining of hypothalamus, pituitary, pineal, ovary, oviduct and uterus samples. (B) Intracellular localization analysis of FST protein in different tissues. (C) Intracellular localization analysis of NR5A1 protein in different tissues. (D) Intracellular localization analysis of PRL protein in different tissues.

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